PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
DRP 016 - PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IB 99/01957	08/12/1999	08/12/1998
Applicant		
DEBIO RECHERCHE PHARMACEUTIQUE S.A. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

[ ] as suggested by the applicant.

[X] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

INTERNATIONAL SEARCH REPORT

[redacted]

International Application No
P[redacted]B 99/01957

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48 C07K1/107

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 01758 A (DEBIOPHARM SA) 21 February 1991 (1991-02-21) the whole document & US 5 286 637 A cited in the application ---	1-5,11
X	WO 92 20362 A (V SHASHOUA) 26 November 1992 (1992-11-26) page 26 ---	1-5,11
X	F LAPICQUE & E DELLACHERIE: "Polysaccharide prodrugs for enzymatically controlled release" JOURNAL OF CONTROLLED RELEASE., vol. 4, 1986, pages 39-45, XP002105708 AMSTERDAM NL the whole document --- -/--	1-5,11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 2000	05/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Masturzo, P

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]IB 99/01957

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	A DE MARRE ET AL.: "Evaluation of the hydrolytic and enzymatic stability of macromolecular Mitomycin C derivatives" JOURNAL OF CONTROLLED RELEASE., vol. 31, 1994, pages 89-97, XP000456583 AMSTERDAM NL the whole document	1-11
X	EP 0 536 671 A (HOECHST AG) 14 April 1993 (1993-04-14) the whole document	12

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/01957

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9101758	A	21-02-1991	AT	118686 T	15-03-1995
			CA	2038935 A,C	08-02-1991
			DE	69017180 D	30-03-1995
			DE	69017180 T	20-07-1995
			DK	437563 T	29-05-1995
			EP	0437563 A	24-07-1991
			ES	2069082 T	01-05-1995
			JP	2662311 B	08-10-1997
			JP	4501121 T	27-02-1992
			US	5286637 A	15-02-1994
WO 9220362	A	26-11-1992	NONE		
EP 536671	A	14-04-1993	CA	2079921 A	08-04-1993
			DE	59205565 D	11-04-1996
			DK	536671 T	12-08-1996
			JP	5221941 A	31-08-1993
			US	5439806 A	08-08-1995
			US	5639859 A	17-06-1997

INTERNATIONAL SEARCH REPORT

Intern[redacted]l Application No
PCT/[redacted] 99/01957

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48 C07K1/107

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 01758 A (DEBIOPHARM SA) 21 February 1991 (1991-02-21) the whole document & US 5 286 637 A cited in the application	1-5,11
X	WO 92 20362 A (V SHASHOUA) 26 November 1992 (1992-11-26) page 26	1-5,11
X	F LAPICQUE & E DELLACHERIE: "Polysaccharide prodrugs for enzymatically controlled release" JOURNAL OF CONTROLLED RELEASE., vol. 4, 1986, pages 39-45, XP002105708 AMSTERDAM NL the whole document	1-5,11

-/—

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 2000	05/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Masturzo, P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intel[redacted]l Application No
PCT/IB 99/01957

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	A DE MARRE ET AL.: "Evaluation of the hydrolytic and enzymatic stability of macromolecular Mitomycin C derivatives" JOURNAL OF CONTROLLED RELEASE., vol. 31, 1994, pages 89-97, XP000456583 AMSTERDAM NL the whole document	1-11
X	EP 0 536 671 A (HOECHST AG) 14 April 1993 (1993-04-14) the whole document	12

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

[redacted] patent family members

Inter[redacted] Application No
PCT/[redacted]99/01957

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9101758 A	21-02-1991	AT 118686 T	15-03-1995
		CA 2038935 A,C	08-02-1991
		DE 69017180 D	30-03-1995
		DE 69017180 T	20-07-1995
		DK 437563 T	29-05-1995
		EP 0437563 A	24-07-1991
		ES 2069082 T	01-05-1995
		JP 2662311 B	08-10-1997
		JP 4501121 T	27-02-1992
		US 5286637 A	15-02-1994
WO 9220362 A	26-11-1992	NONE	
EP 536671 A	14-04-1993	CA 2079921 A	08-04-1993
		DE 59205565 D	11-04-1996
		DK 536671 T	12-08-1996
		JP 5221941 A	31-08-1993
		US 5439806 A	08-08-1995
		US 5639859 A	17-06-1997

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 11 August 2000 (11.08.00)	
International application No. PCT/IB99/01957	**Applicant's or agent's file reference** DRP 016 - PCT
International filing date (day/month/year) 08 December 1999 (08.12.99)	**Priority date** (day/month/year) 08 December 1998 (08.12.98)
Applicant SCHIAVON, Oddone et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

07 July 2000 (07.07.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Zakaria EL KHODARY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A61K 47/48, C07K 1/107

A1

(11) International Publication Number: WO 00/33881

(43) International Publication Date: 15 June 2000 (15.06.00)

(21) International Application Number: PCT/IB99/01957

(22) International Filing Date: 8 December 1999 (08.12.99)

(30) Priority Data:
98123307.5 8 December 1998 (08.12.98) EP

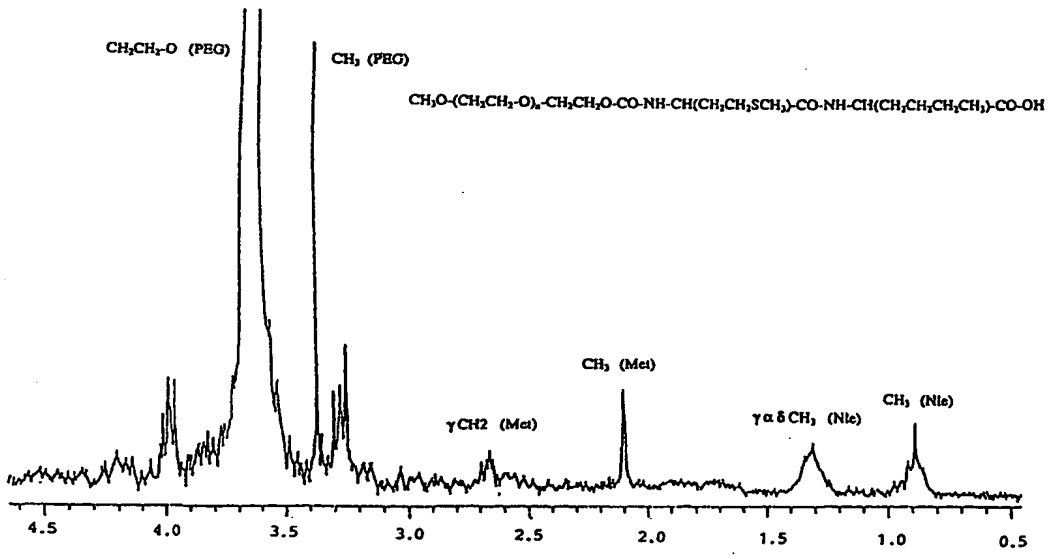
(71) Applicant *(for all designated States except US)*: DEBIO RECHERCHE PHARMACEUTIQUE S.A. [CH/CH]; 146, rue du Levant, CH–1920 Martigny (CH).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SCHIAVON, Oddone [IT/IT]; Via E. Zago, 13, I–35128 Padova (IT). VERONESE, Francesco [IT/IT]; University of Padova, Department of Pharmaceutical Sciences, Via F. Marzolo, 5, I–35131 Padova (IT). CALICETI, Paolo [IT/IT]; Via Gattamelata, 156/c, I–35128 Padova (IT). ORSOLINI, Piero [IT/CH]; 146, route du Levant, Case Postale 368, CH–1920 Martigny (CH).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: BIOLOGICALLY ACTIVE CONJUGATES HAVING A DETECTABLE REPORTER MOIETY AND METHOD OF IDENTIFICATION OF SAID DERIVATIVE



(57) Abstract

The aim of the invention is to provide a new method for identifying or analysing polymer linkage sites on macromolecules using amino acid reporter binding. Another aim of this invention is to provide a compound FE – L – M, where M is a molecule consisting of proteins, peptides or polypeptides, FE is a functionalizing entity and L is a linking arm that is stable under physiological conditions but cleavable by specific and selective physical–chemical means.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
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CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
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DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

Inter[illegible] Application No
PCT/IB 99/01957

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48 C07K1/107

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 92 20362 A (V SHASHOUA) 26 November 1992 (1992-11-26) page 26	1-5,11
X	F LAPICQUE & E DELLACHERIE: "Polysaccharide prodrugs for enzymatically controlled release" JOURNAL OF CONTROLLED RELEASE., vol. 4, 1986, pages 39-45, XP002105708 AMSTERDAM NL the whole document	1-5,11

-/—

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 2000	05/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Masturzo, P

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	A DE MARRE ET AL.: "Evaluation of the hydrolytic and enzymatic stability of macromolecular Mitomycin C derivatives" JOURNAL OF CONTROLLED RELEASE., vol. 31, 1994, pages 89-97, XP000456583 AMSTERDAM NL the whole document	1-11
X	EP 0 536 671 A (HOECHST AG) 14 April 1993 (1993-04-14) the whole document	12

INTERNATIONAL RCH REPORT

nformation on patent family members

Inter Application No
PCT/IB 99/01957

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9101758	A	21-02-1991	AT	118686 T	15-03-1995
			CA	2038935 A,C	08-02-1991
			DE	69017180 D	30-03-1995
			DE	69017180 T	20-07-1995
			DK	437563 T	29-05-1995
			EP	0437563 A	24-07-1991
			ES	2069082 T	01-05-1995
			JP	2662311 B	08-10-1997
			JP	4501121 T	27-02-1992
			US	5286637 A	15-02-1994
WO 9220362	A	26-11-1992	NONE		
EP 536671	A	14-04-1993	CA	2079921 A	08-04-1993
			DE	59205565 D	11-04-1996
			DK	536671 T	12-08-1996
			JP	5221941 A	31-08-1993
			US	5439806 A	08-08-1995
			US	5639859 A	17-06-1997

TENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
DRP 016 - PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB99/01957	08/12/1999	08/12/1998
International Patent Classification (IPC) or national classification and IPC A61K47/48		
Applicant DEBIO RECHERCHE PHARMACEUTIQUE S.A. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 9 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
07/07/2000	19.03.2001
Name and mailing address of the international preliminary examining authority: European Patent Office - Gitschiner Str. 103 D-10958 Berlin Tel. +49 30 25901 - 0 Fax: +49 30 25901 - 840	Authorized officer Korsner, S-E Telephone No. +49 30 25901 329

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IB99/01957

I. Basis of the rep rt

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-12,19-21,23, 24	as originally filed			
22	as received on	14/07/2000	with letter of	07/07/2000
13-18	with telefax of	06/02/2001		

Claims, No.:

1-8	with telefax of	06/02/2001

Drawings, sheets:

1/6-6/6	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-8 (but see VIII:3)
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-8 (but see VIII:3)
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-8
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

V. Reasoned Statement

Initial note:

The amendments requested with the Applicant's letter of 07.07.00 have been accepted; i.e. on original page 22, lines 13 and 15, "Gln-Gly" has been replaced by "Asp-Pro".

The following document will be referred to in this report:

D1 = WO - A - 9101758

1. Novelty (Article 33(2) PCT)

The drug polymer derivatives disclosed in the Applicant's earlier application, D1, consist of proteins linked to e.g. PEG via a linker that can be a residue of 1-3 amino acids; see for instance claims 5-6 of D1.

The present application provides a selection of dipeptide linkers and this subject-matter (Claims 1-7) can therefore be seen as novel (by selection) over said document and any other cited prior art.

The intermediates of Claim 8 are also considered novel over the prior art for the same reason.

2. Inventive step (Article 33(3) PCT)

As discussed on page 9, the use of these selected dipeptides results in M-bound reporter moieties that are very stable to acid hydrolysis and easily identified by a later analysis.
See also the examples.

This could not be derived from the cited prior art and the inventive step is therefore acknowledged.

VIII. Certain observations

Claims:

1.
The definitions of Claim 1 could be simplified, e.g.

A biological active conjugate of formula (I):
FE-L-M, where

M = a peptide, polypeptide or protein,
L = a linker comprising a dipeptide selected from Met-Nle, Met-ßAla, Gln-Gly or Asp-Pro
FE = PEG, PVP, PacM, dextran, hormones, antibodies or antibody fragments

2.
Claim 7 appears somewhat unclear; it could possibly be simplified by first referring to FE, L and M without definitions, and then add, at the end of the claim, "where FE, L and M are defined as in Claim 1".

3.
Owing to the term "comprising" in the definitions of the linkers, said linkers are not restricted to dipeptides (but cover any longer peptide including these dipeptide fragments).

In so far as FE, L and M are all peptide sequences (e.g. antibody fragment + linker + peptide/protein), the claims cover quite undefined polypeptides/ proteins.

This question may have to be reviewed at a later national/regional phase.
Note that FE = PEG in all examples.

Description:

4.
The Description has to be adapted to the amended claims, it should not give the impression that FE, L or M have broader definitions than those given in the amended claims.

NB.
All observations are intended for consideration in any later phase.

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Fig. 4 a) is the HPLC elution of (MPEG - Met - Nle)$_2$ - insulin; b) being the HPLC elution of (Nle)$_2$ - insulin obtained from CNBr cleavage of (MPEG - Met - Nle)$_2$ - insulin;

Fig. 5 a) is the Maldi mass spectrum of Nle - insulin obtained from CNBr cleavage of MPEG - Met - Nle-insulin, b) being the Maldi mass spectrum of (Nle)$_2$ - insulin obtained from CNBr cleavage of (MPEG - Met - Nle)$_2$ - insulin,

Fig. 6 is the chromatographic profile of a) native lysozime, b) being MPEG - Met - Nle - conjugate lysozime, c) being Nle - lysozime obtained from CNBr cleavage of MPEG - Met - Nle - conjugate lysozime.

Example 1. Synthesis of MPEG-Met-Val-OH (5000 Da)

MPEG - OH 5000 (where MPEG is Poly(ethylene glycol) methyl ether), dissolved in anhydrous methylene chloride, was reacted with 4-nitrophenyl chloroformate at pH 8 in the presence of equimolecular amount of triethylammine to give MPEG-*p*-nitrophenylcarbonate. 1 g of the product (0. 19 mmol) was added in small portions to 283 mg (1. 14 mmol) of H-Met-Val-OH dissolved in 4 ml of a 50% acetonitrile solution in water and brought to pH 8 with TEA. After overnight stirring, the pH of the reaction mixture was adjusted to 3 using solid citric acid; 4-nitrophenol was removed by ether extraction while the product was later extracted by chloroform. The organic solution was anhydrified with solid dry Na_2SO_4 and concentrated under reduced pressure. After the addition of 200 ml of diethyl ether to the chloroform, the solution was cooled at –20°C for 1 hour and the separated product was collected by filtration and dried under vacuum. The MPEG-Met-Val-OH was further purified by ionic exchange chromatography on a QAE-Sephadex A50 column (2.5x55 cm) eluted first with water and then with a water solution of NaCl 10 mM. The eluted fractions were analysed by iodine assay to detect PEG. The fractions containing the sodium salt of MPEG-peptide were collected, acidificated and lyophilised. The yield was 70-75% in MPEG-peptide. The product was identified by ^{1}H-NMR (200 MHz; $CDCl_3$).

Example 2. Synthesis of MPEG-Met-Nle-OH (5000 Da)

MPEG 5000 was bound to Met-OH to obtain MPEG-Met-OH following the method described in example 1 in the preparation of MPEG-Met-Val-OH. 523 mg (2.88 mmol) of H-Nle-OMe and a equimolecular amount of TEA (401 µl) were added to a solution of 10 ml of anhydrous dichloromethane, containing 3.71 g (0.72 mmol) of the synthesised MPEG-Met-OH, 305 mg (0.72 mmol) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimidemetho-*p*-toluenesulfonate (CMC) and 97 mg (0.72 mmol) of 1-hydroxybenzotriazole (HOBt). After stirring for 3 days at room temperature, the mixture was dropped into 250 ml of diethyl ether; the precipitate was collected by filtration and dried under vacuum. The product was further purified by ionic exchange chromatography on a QAE-Sephadex A50 column (2.5x55 cm) eluted first with water and then with a water solution of NaCl 10 mM. The fractions were analysed by iodine assay to reveal the presence of PEG. The fractions containing the MPEG-peptide-methyl ester (MPEG-Met-Nle-OMe), were collected and lyophilised. The yield was 93%. The structure of the product was verified by ^{1}H-NMR (200 MHz; $CDCl_3$).

The methyl ester of Nle was hydrolysed in a solution of methanol containing 20 equivalents of NaOH 1N, left stirring the mixture for 2 days at room temperature. The solution pH was brought to 2 using HCl lN and the product extracted into chloroform. The chloroform solution was anhydrified with solid dry Na_2SO_4 and concentrated under reduced pressure. The addition of 200 ml of diethyl ether, under vigorous stirring, yielded a solid product that was collected by filtration and dried under vacuum. The yield was 67% in MPEG-Met-Nle-OH. The product identity was verified by ^{1}H-NMR (200 MHz, $CDC1_3$). The spectrum is reported in Fig. 1.

Example 3. Synthesis of MPEG-Met-Nle-OH (10000 Da)

The product was prepared according to example 2 but starting from a MPEG-OH of 10000 MW.

Example 4. Synthesis of (MPEG-Met-Nle)$_2$-nonapeptide

The MPEG-Met-Nle-OH terminal COOH was activated as hydroxysuccinimidil ester (MPEG-Met-Nle-OSu) according to a method already reported in literature for MPEG with Nle as aminoacid spacer *(Applied Biochemistry and Biotechnology, 27, 45-54, 1991).*

Totally protected nonapeptide obtained by solid phase synthesis with the following structure Nps-Thr(*t*Bu)-Ser(tBu)-Asp(O*t*Bu)-Tyr(*t*Bu)-Ser(*t*Bu)-Lys-(Boc)-Tyr(*t*Bu)-Leu-Asp(O*t*Bu)-OH was deprotected maintaining it in a 1:1 mixture of trifluoroacetic acid and dichloromethane, for 2 hours at room temperature. The solution was concentrated to dryness and the Nps group removed by repeated extractions in diethyl ether at 0°C. The product was finally dried under vacuum for 12 hours over KOH. The yield of the totally deprotected peptide was 90%.

43 mg (4.0 µmol) of MPEG-Met-Nle-OSu were added, in small portions, to 0.9 mg (0.8 µmol) of the deprotected nonapeptide dissolved in 1 ml of DMSO, at pH 8 with TEA. (nonapeptide : MPEG-Met-Nle-OSu molar ratio = 1: 5). After 3 days stirring at room temperature, the pH of the reaction mixture was adjusted to 3 using HCl lN and the product was extracted many times into chloroform. The chloroform solution was anhydrified with solid dry Na_2S0_4 and concentrated to dryness. The conjugate was further purified by means of a Shimadzu semi-preparative HPLC system, using a reverse-phase Vydac 218TP54 column (C_{18} bonded-phase, 0.46x25 cm i.d, 5 µm particle diameter) and a linear gradient of aqueous 0.05% trifluoroacetic acid and 0.05% trifluoroacetic acid in acetonitrile. The absorption at 280 nm wavelength was monitored. The product was characterised by iodine assay for MPEG and aminoacid analysis.

Example 5. Synthesis of MPEG-Met-Nle-insulin

8.6 mg (1 µmol) of MPEG-Met-Nle-OSu prepared as in example 4, were added to 6 mg (1 µmol) of bovine insulin dissolved in 1 ml of DMSO (amino groups of insulin: MPEG molar ratio = 3: 1) and the mixture

was left stirring for 5 hours at room temperature. The conjugate was purified by means of a Shimadzu preparative HPLC system, using a reverse-phase Vydac 218TP1022 column (C_{18} bonded-phase, 2.2x25 cm i.d., 10 um particle diameter) and a linear gradient of aqueous 0.05% trifluoroacetic acid and 0.05% trifluoroacetic acid in acetonitrile, monitoring the absorption at 280 nm wavelength. The fractions of the main elution peak of conjugated insulin were collected and lyophilised. The identity of the product was evaluated by amino groups colorimetric assay that indicated the loss of one amino residue in insulin and by aminoacid analysis after acid hydrolysis that revealed one Nle per insulin, again in favour of binding of a single polymer chain per insulin molecule.

Example 6. Synthesis of (MPEG-Met-Nle)$_2$-insulin

17 mg (2 μmol) of MPEG-Met-Nle-OSu prepared as in example 4, were added to 6 mg (1 μmol) of bovine insulin dissolved in 1 ml of DMSO (amino groups of insulin : MPEG molar ratio = 3:2). After stirring for 5 hours at room temperature, the product was purified by reverse-phase HPLC as described in example 5 for MPEG-Met-Nle-insulin. The fractions of the main elution peak of conjugated insulin were collected and lyophilised. The identity of the conjugate was evaluated by amino groups colorimetric assay that indicated the loss of two amino residues and by aminoacid analysis after acid hydrolysis that demonstrated the presence of two Nle per insulin, indicating the binding of two PEG moiety per insulin molecule.

Example 7. Reaction of (MPEG-Met-Nle)$_2$-nonapeptide with CNBr

1 mg (85.2 μmol) of (MPEG-Met-Nle)$_2$-nonapeptide, obtained as described in example 4 was treated with 100 equivalents of CNBr in aqueous 70% formic acid (according to *Methods in Enzymology*, pag. 238-254, 1967). After 24 hours standing at room temperature, the mixture was poured into 10 volumes of water and lyophilised. (the procedure was repeated twice). The obtained products were analysed by means of a Shimadzu analytical HPLC system, using a reverse-phase Vydac 218TP54 column (C_{18} bonded-phase, 0.46x25 cm i.d., 5 um particle

diameter) and a linear gradient of aqueous 0.05% trifluoroacetic acid and 0.05% trifluoroacetic acid in acetonitrile. The elution pattern indicated a shift in the elution times from 36.3 min- for the PEG-peptide conjugate, to 15.8 min. for the same product upon treatment with CNBr. This value was close to that of the peptide alone (16.4 min.) demonstrating that the PEG moiety was removed. Aminoacid analysis after acid hydrolysis and mass spectrometry revealed the presence of two Nle residues per peptide molecule, indicating the validity of this method in removing PEG from the molecule leaving Me as reported group bound to the polypeptide.

Example 8. Evaluation of the number of polymer chains bound to differently modified insulin samples after polymer removal

The products obtained as described in example 5 and 6 corresponding to insulin conjugates with one or two chains of MPEG-Met-Nle respectively, were treated with CNBr in aqueous 70% formic acid. The molar ratio between insulin content and CNBr was 1 to 200. After 24 hours stirring at room temperature, the mixture was poured into 10 volumes of water and lyophilised and the procedure was repeated twice. The obtained products were analysed by means of a Shimadzu analytical HPLC system, using a reverse-phase Vydac 218TP54 column (C_{18} bonded-phase, 0.46x25 cm i.d., 5 um particle diameter) and a linear gradient of aqueous 0.05% trifluoroacetic acid and 0.05% trifluoroacetic acid in acetonitrile. The elution pattern indicated a shift in the elution times from the 22.2 min. and the 23.2 min. for the PEG-insulin conjugates with one or two PEG chains respectively, to the 18.9 min. and the 19.2 min. for the products after CNBr cleavage. These values that are close to that of insulin alone (18.3 min.), demonstrated that the PEG moiety was removed. (see Fig. 2, 3 e 4) The mass spectrometry analysis revealed a mass of one insulin molecule plus one Me residue when starting from the monoderivatized insulin (MPEG-Met-Nle-insulin) and plus two residues in the case of $(MPEG\text{-}Met\text{-}Nle)_2$-insulin. (see Fig. 5) The mass spectra patterns were by far more clear of those obtained in the analysis of the conjugates (examples 5 and 6) since the presence of the polymer, with its polydispersivity, makes the spectra

and their interpretation more difficult and uncertain. Furthermore these values were confirmed by aminoacid analysis, finding that indicated one or two Nle residues present per insulin molecule in the samples.

All these data are in favour of the possibility to remove PEG from the molecule leaving Nle bound 10 and thus demonstrating the validity of the method.

Example 9. Reduction and carboxymethylation of Nle-insulin

500 µg (0.08 µmol) of Nle-insulin, obtained from MPEG-Met-Nle-insulin as described in example 8, were dissolved in 250 µl of TRIS-HCI buffer, pH 7.5, containing 2 mM EDTA and 6 M Gdn-HCl 3.8 ing of 1,4-dithio-L-threitol (DTT) were added and the mixture was left standing for 2 hours at 37°C. Finally 9.2 mg of iodoacetamide were added and the mixture was left for 1 hour at 37°C. The solution was lyophilised and the products fractionated by means of a Shimadzu semi-preparative HPLC system, using a reverse-phase Vydac 218TP54 column (C_{18} bonded-phase, 0.46x25 cm Ld., 5 um particle diameter) with a linear gradient of aqueous 0.05% trifluoroacetic acid and 0.05% trifluoroacetic acid in acetonitrile. The eluted products monitored by absorption at 226 nm wavelength, revealed the presence of two main peaks, corresponding to chain α and β of insulin, that were separated and recovered. The aminoacid analysis after acid hydrolysis revealed Nle as additional aminoacid among those of chain β peak. The Edman degradation (according to *Protein Practical Chemistry*, pag. 371-373, 1986) released Phe and Nle at the first step, indicating that the α-amino group was free and consequently PEG was bound to ^{29}Lys of chain β. Furthermore mass spectrometry analysis demonstrated that the increase of one Nle mass was present only in chain β.

Example 10. Reduction and carboxymethylation of Nle_2-insulin

600 µg (0. 1 µmol) of Nle_2-insulin, obtained from MPEG-Met-Nle-insulin as described in example 8, were dissolved in 200 µl of TRIS-HCl buffer, pH 7.5, containing 2 mM EDTA and 6 M

using the activated ester of Example 17. This conjugate demonstrated biological activities and was able to be selectively cleaved at the Gln-Gly bound applying a classical hydrazine treatment.

Example 19

According to a similar procedure as the one described in Example 1, MPEG-Asp-Pro-OH has been prepared starting from MPEG-OH 5000 and H- Asp-Pro -OH and then transformed into its succinimide activated ester MPEG- Asp-Pro -OSu.

Example 20

According to a similar procedure as the one described in Example 5, the biological active conjugate MPEG-Gln-Gly-insulin has been obtained using the activated ester of Example 19. This conjugate demonstrated biological activities and was able to be selectively cleaved at the Gln-Gly bound applying a classical by mild acid treatment.

Claims

1. A biological active conjugate derivative having the following general formula (I)

FE - L - M (I);

where :

M represents the corresponding radical of a biological active molecule selected from the group consisting of proteins, peptides and polypeptides;

FE represents a functionalizing entity; and

L represents a linking arm having a reporter moiety detectable by standard analytical methods;

characterised in that said linking arm is able to be cleaved by chemical or enzymatic *in vitro* treatment and in that said reporter moiety remains attached to said biological active molecule once the linking arm has been cleaved.

2. The biological active conjugate derivative according to claim 1 characterised in that said functionalizing entity FE is selected from PEG, PVP, PacM, dextran, hormones, antibodies or antibody fragments.

3. The biological active conjugate derivative according any of the preceding claims characterised in that said functionalizing entity FE is a polymer with a molecular weight in the range of 2 Kd to 50 Kd.

4. The biological active conjugate derivative according to claim 3 characterised in that said functionalizing entity FE is a linear polymer.

5. The biological active conjugate derivative according to claim 3 characterised in that said functionalizing entity FE is a branched polymer.

6. The biological active conjugate derivative according to any of the preceding claims characterised in that said linking arm L comprises the following fragment Met –X in which X represents said reporter moiety, said reporter moiety being an amino acid.

7. The biological active conjugate derivative according to claim 6 characterised in that said amino acid X is selected from Nle and beta Ala.

8. The biological active conjugate derivative according to any of claims 1 to 5 characterised in that said linking arm L comprises the dipeptide Gln – Gly.

9. The biological active conjugate derivative according to any of claims 1 to 5 characterised in that said linking arm L comprises the dipeptide Asp – Pro.

10. The biological active conjugate derivative according to any of the preceding claims characterised in that said biological active molecule is a protein selected from insulin, lysozime, interferon, erithropoietin, G-CSF, GH.

11. A method for identifying, on the biological active drug conjugate derivative of claim 1, linkage sites of conjugation of the functionalizing entity FE along the biological active molecule M comprising a specific chemical or enzymatic *in-vitro* cleavage of the linking arm L, releasing, removing and separating FE by classical methods.

12. An intermediate compound, for the preparation of the biological active conjugate of claim 1, having the following general formula (II)

FE - L (II);

where :

FE represents a functionalizing entity; and

L represents a linking arm having a reporter moiety detectable by standard analytical methods.